**TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 USC 371**

| | |
|---|---|
| International Application No.: | PCT/BE99/00105 |
| International Filing Date: | August 14, 1999 |
| Priority Date Claimed: | August 14, 1998 |
| Title of Invention: | NUCLEOTIDE AND/OR AMINO-ACID SEQUENCE CONTROLLING THE EXPRESSION OF A XYLANASE PROMOTER-OPERATOR NUCLEOTIDE SEQUENCE |
| Applicant(s) for DO/EO/US: | Fabrizio Giannotta, Sebastien Rigali, Jean Dusart |

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. (X) This is a **FIRST** submission of items concerning a filing under 35 USC 371.

2. (X) This express request to begin national examination procedures (35 USC 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 USC 371(b) and PCT Articles 22 and 39(1).

3. (X) A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

4. (X) A copy of the International Application as filed (35 USC 371(c)(2))

   a) () is transmitted herewith (required only if not transmitted by the International Bureau).
   b) (X) has been transmitted by the International Bureau.
   c) () is not required, as the application was filed in the United States Receiving Office (RO/US).

5. (X) Amendments to the claims of the International Application under PCT Article 19 (35 USC 371(c)(3))

   a) () are transmitted herewith (required only if not transmitted by the International Bureau).
   b) () have been transmitted by the International Bureau.
   c) () have not been made; however, the time limit for making such amendments has NOT expired.
   d) (X) have not been made and will not be made.

6. (X) An oath or declaration of the inventor(s) (35 USC 371(c)(4)).

7. (X) A copy of the International Preliminary Examination Report with any annexes thereto, such as any amendments made under PCT Article 34.

8. (X) An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

9. (X) A FIRST preliminary amendment.

10. (X) International Application as published:
    a. (X) Publication Cover Sheet
    b. (X) 15 pages of disclosure
    c. (X) Sequence Listing in 5 pages
    d. (X) 3 pages of drawings
    e. (X) International Search Report

11. (X) Small Entity status is established.

12. (X) PCT Form PCT/IPEA/402.

13. (X) PCT Form PCT/IB/308.

14. (X) PCT request form.

15. (X) A return prepaid postcard.

16. (X) The following fees are submitted:

| | | | | **FEES** |
|---|---|---|---|---|
| | **BASIC FEE** | | | $860 |
| **CLAIMS** | **NUMBER FILED** | **NUMBER EXTRA** | **RATE** | |
| Total Claims | 26 - 20 = | 6 × | $18 | $108 |
| Independent Claims | 1 - 3 = | 0 × | $80 | $0 |
| | **TOTAL OF ABOVE CALCULATIONS** | | $968 | |
| Reduction by 1/2 for filing by small entity (if applicable). Verified Small Entity statement must also be filed. (NOTE 37 CFR 1.9, 1.27, 1.28) | | | | $484 |
| | **TOTAL NATIONAL FEE** | | | $484 |
| | **TOTAL FEES ENCLOSED** | | | $484 |

17. (X) A check in the amount of $484 to cover the above fees is enclosed.

18. (X) Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). $40 per property.

19. (X) The Commissioner is hereby authorized to charge only those additional fees which may be required, now or in the future, to avoid abandonment of the application, or credit any overpayment to Deposit Account No. 11-1410. A duplicate copy of this sheet is enclosed.

**NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.**

SEND ALL CORRESPONDENCE TO:

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VANM201.001APC

PATENT

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

| | | | |
|---|---|---|---|
| Applicant | : | Giannotta, et al | Group Art Unit unknown |
| Int'l Appl. No. | : | PCT/BE99/00105 | |
| Int'l Filing Date | : | August 14, 1999 | |
| For | : | NUCLEOTIDE AND/OR AMINO-ACID SEQUENCE CONTROLLING THE EXPRESSION OF A XYLANASE PROMOTER-OPERATOR NUCLEOTIDE SEQUENCE | |
| Examiner | : | Unknown | |

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

Preliminary to Examination on the merits, please amend the above-captioned patent application as follows:

**IN THE SPECIFICATION**

On page 1 of the Specification, line 12, after the Title of the Invention, please insert --This is the U.S. National Phase of International Application PCT/BE99/00105, filed August 12, 1999, which claims priority of U.S. Provisional Application, 60/096,556, filed August 14, 1998.--.

On page 3, line 31, please cancel the word "pairs" and substitute in its place --pair--.

On page 5, line 10, please cancel the word "hybridisation" and substitute in its place --hybridization--.

On page 5, line 15, please cancel the word "hybridisation" and substitute in its place --hybridization--.

On page 5, line 16, please cancel the word "hybridisation" and substitute in its place --hybridization--.

On page 5, line 20, please cancel the word "hybridised" and substitute in its place --hybridized--.

On page 5, line 22, please cancel the word "hybridisation" and substitute in its place --hybridization--.

On page 6, line 9, after the word "described.", please insert --[SEQ ID NO:4]--.

On page 9, line 2, in Table 1, after the sequence "CGAAACTGTTGA", please insert --[SEQ ID NO:7]--.

On page 9, line 3, in Table 1, after the sequence "TTTCCGAAAGTTTGCC", please insert --[SEQ ID NO:8]--.

On page 9, line 4, in Table 1, after the sequence "TCGAAACTTTCG", please insert --[SEQ ID NO:9]--.

On page 9, line 5, in Table 1, after the sequence "t CGAAA g c c", please insert --[SEQ ID NO:10]--.

On page 9, line 8, after the word, "sequence", please insert --TXXCGAAAXXGXCXC[SEQ ID NO:10]--.

On page 11, line 15, please cancel the word "biotine" and substitute in its place --biotin--.

On page 11, line 16, please cancel the word "hybridisation" and substitute in its place --hybridization--.

On page 12, line 25, after the word "fragment", please cancel the word "SEQ ID NO 5:" and substitute in its place --SEQ ID NO:6--.

On page 12, line 28, after the sequence, please cancel "(or SEQ ID NO 4)" and substitute in its place --(or SEQ ID NO:5)--.

On page 12, line 32, please cance the words "streptomycine/spectinomycine" and substitute in its place --streptomycin/spectinomycin--.

On page 13, line 19, please cancel the word "foreigner" and substitute in its place --foreign--.

On page 14, line 1, please cancel the word "CLAIMS" and substitute in its place –WHAT IS CLAIMED IS:--.

**IN THE CLAIMS**

**Please amend the claims as follows:**

1. **(Amended) [Isolated and purified genetic sequence (1) controlling in trans]**An isolated polynucleotide which controls the expression of a xylanase promoter-operator nucleotide sequence in trans, comprising **[(2)]** at least about 100 nucleotides of SEQ ID NO:1, its complement, or a homolog, wherein said homolog controls the expression of said xylanase promoter-operator.

2. **(Amended) [Isolated and purified genetic sequence]**The isolated polynucleotide according to claim 1, **[being a nucleotide sequence which presents]**with more than 60% homology with the nucleotide sequence SEQ ID NO:1 or its complementary strand.

3. **(Amended) [Isolated and purified genetic sequence]**The isolated polynucleotide genetic sequence according to claim 2, **[which presents]**with more than 80%**[, preferably more than 90%, more specifically more than 95%,]** homology with the nucleotide sequence SEQ ID NO:1 or its complementary strand.

4. **(Amended) [Isolated and purified genetic sequence]**The isolated polynucleotide according to claim 1, **[any one of the preceding claims, being the nucleotide sequence SEQ ID NO:1, its complementary strand or a portion thereof having more than 100 nucleotides and]**wherein said polynucleotide **[encoding]**encodes a peptide **[controlling]**which positively and/or negatively controls the activation of a xylanase promoter-operator nucleotide sequence.

5. **(Amended) [Isolated and purified genetic sequence]**The isolated polynucleotide according to claim 1, **[being]**encoding an amino-acid sequence **[which presents]**having more than 60% homology with SEQ ID NO:2.

6. **(Amended) [Isolated and purified genetic sequence]**The isolated polynucleotide according to claim 5, **[being]**wherein the amino-acid sequence

**[which]** presents more than 80%**[, preferably more than 90%, more specificalloy more than 95%,]** homology with SEQ ID NO:2.

7. **(Amended) [Isolated and purified genetic sequence]**The isolated polynucleotide according to claim 1, **[being]**encoding the amino-acid sequence SEQ ID NO 2 or a portion thereof having more than 50 amino-acids **[which is capable of controlling]**wherein said portion controls positively and/or negatively in trans the expression of a xylanase promoter-operator nucleotide sequence.

8. **(Amended) [Nucleotide]**A polynucleotide construct **[(6)]** comprising the isolated and purified polynucleotide **[sequence]** according to **[any one of the claims 1 to 4]**claim 1, operably linked to a xylanase promoter-operator poynucleotide **[sequence (2) and possibly a nucleotide sequence (5) which is cis-activated by said xylanase promoter-operator nucleotide sequence (2)]**.

9. **(Amended) [Vector (7), preferably a plasmid,]**A vector comprising the isolated and purified polynucleotide **[sequence (2)]** according to **[any one of the claims 1 to 7 or the nucleotide construct (6) according to claim 8]** claim 1.

10. **(Amended) [Cell]**A cell transformed by the vector according to claim 9 **[and which allows the expression of the isolated and purified genetic sequence according to any one of the claims 1 to 7]**.

**Please add the following Claims:**

11. The isolated polynucleotide according to claim 3, with more than 90% homology with the nucleotide sequence SEQ ID NO:1 or its complementary strand.

12. The isolated genetic sequence according to claim 3, with more than 95% homology with the nucleotide sequence SEQ ID NO:1 or its complementary strand.

13. The isolated polynucleotide of Claim 1 further comprising a cofactor.

14. The isolated polynucleotide of Claim 11 wherein said cofactor is selected from the group consisting of glucose, xylan, and mixtures thereof.

15. A method for the up-regulation or down-regulation of xylanase, comprising: providing the polynucleotide of Claim 1 to a cell which expresses xylanase.

16. The method of Claim 15, further comprising providing a cofactor.

17. The method of Claim 16, wherein said cofactor is selected from the group consisting of glucose, xylan, and mixtures thereof.

18. The polynucleotide of Claim 1 wherein said xylanase promoter-operator is SEQ ID NO:2.

19. The polynucleotide of Claim 1 wherein said xylanase promoter-operator is from Streptomyces strain EC3 xlnC.

20. A method for the production of antibiotics, malting of cereals, and production of paper, comprising

adding the polynucleotide of Claim 1 or the polypeptide encoded by the polynucleotide of Claim 1 to any cell which is producing any substance selected from the group consisting of: antibiotics, enzymes for malting of cereals, and enzymes for the production of paper.

21. The isolated polynucleotide according to claim 5, wherein the amino-acid sequence presents more than 90% homology with SEQ ID NO:2.

22. The isolated polynucleotide according to claim 5, wherein the amino-acid sequence presents more than 95%, homology with SEQ ID NO:2.

23. The polynucleotide of claim 8, further comprising a polynucleotide which is cis-activated by said xylanase promoter-operator nucleotide sequence.

24. The vector of claim 9 wherein said vector is a plasmid.

25. The isolated polynucleotide of Claim 1 comprising SEQ ID NO:3.

26. The isolated polynucleotide of Claim 5 comprising SEQ ID NO:4.

**IN THE ABSTRACT**

Please add the enclosed abstract page 16.

## REMARKS

The Specification and Claims have been amended to conform to practice before the United States Patent and Trademark Office, to correct minor informalities such as European spellings of words. An abstract, corresponding to that in the PCT application has been added. Claims 11-27 have been added. Support for the added claims can be found in the original Claims as well as in the Specification. Specifically, support for added Claim 19 can be found on page 3, lines 33-34 and page 4, line 1. Support for added Claim 20 can be found on page 7, lines 29-34

through page 8, line 1-3. As a result of the amendments made herein claims 1-27 are pending. No new matter has been added herewith.

**Conclusion**

Please charge any additional fees, including any fees for additional extension of time, or credit overpayment to Deposit Account No. 11-1410.

Respectfully submitted,

KNOBBE, MARTENS, OLSON & BEAR, LLP

Dated: 14 Feb. 2001

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## ABSTRACT

The present invention is related to an isolated and purified genetic sequence controlling in trans the expression of a xylanase promoter-operator nucleotide sequence.

# NUCLEOTIDE AND/OR AMINO-ACID SEQUENCE CONTROLLING THE EXPRESSION OF A XYLANASE PROMOTER-OPERATOR NUCLEOTIDE SEQUENCE

## Field of the invention

The present invention is related to a new nucleotide sequence controlling in trans the expression of a xylanase promoter-operator nucleotide sequence, the amino-acid sequence encoded by said new nucleotide sequence, the vector comprising said new nucleotide sequence and the cell, preferably a *Streptomyces* strain, transformed by said vector.

## Background of the invention

In beer production, efficient hydrolysis of xylans and other saccharides is important because said compounds can be involved in production problems such as wort viscosity (Ducroo, P. & Frelon, P.G., *Proceedings of the European Brewery Convention Congress, Zurich*, 1989, 445; Viëtor, R.J. & Voragen, A.G.J., *Journal of the Institute of Brewing*, 1993, **99**, 243) and filterability and haze formation (Coote N. & Kirsop, B.H., *Journal of the Institute of Brewing*, 1976, **82**, 34; Izawa, M., Kano, Y. & Kanimura, M., *Proceedings Aviemore Conference on Malting, Brewing and Distilling*, 1990, 427).

In other areas, efficient hydrolysis of xylans and/or arabinoxylans is highly desirable as well. Examples include rye and wheat breadmaking processes, paper and pulp technologies (see US patent 5,116,746). It follows that a lot of research efforts have been devoted to the xylan hydrolysis enzymes due to their applications as described above.

**Aims of the present invention**

The aim of the present invention is to provide a method and system which improve the control upon the expression of nucleotide sequence encoding enzymes such as xylanase, as well as homologous or heterologous sequences of said enzymes whose transcription is also activated by a xylanase promoter-operator regulatory sequence.

A specific aim of the present invention is to provide such a method and system for improving enzymatic processes, especially for improving production of antibiotics, malting processes of cereals such as barley, sorghum and wheat, production of beers, of baked or extruded cereals products, animal feed stuff, the production of starch derived from syrups, sorbitol, xylose and xylitol, and for the improvement of paper and pulp technologies.

**Summary of the invention**

The present invention is related to a new nucleotide sequence **1** which controls the expression of any xylanase promoter-operator nucleotide sequence **2**. Said control upon the activation of a xylanase promoter-operator nucleotide sequence **2** is advantageously obtained by trans-activation (said new nucleotide sequence **1** encoding a

trans-activated factor which controls the activation of said xylanase promoter-operator nucleotide sequence 2).

Therefore, the present invention is also related to said factor, preferably a peptidic factor 3 which is an activator and/or repressor encoded by said nucleotide sequence 1 and which controls positively and/or negatively the expression of a xylanase promoter-operator nucleotide sequence 2.

Advantageously, said factor could be present in a composition with other cofactors 4 that induce positively and/or negatively said mechanism.

Preferably, said cofactors 4 present in said composition are selected from the group consisting of glucose, xylan or a mixture thereof.

The Inventors have discovered unexpectedly that the presence of glucose induces a repressive mechanism upon the activation of a xylanase promoter-operator nucleotide sequence, while the presence of xylan induces a positive mechanism of said expression. The simultaneous presence of said two cofactors in a medium induces also positively the expression of a xylanase promoter-operator nucleotide sequence.

It is meant by "a xylanase promoter-operator nucleotide sequence", any nucleotide sequence 2 which cis-activates any nucleotide sequence 5 encoding a xylanase enzyme.

A classification of the xylanase enzymes in the categories F/10 and G/11 is described by Henrissart et al. (*Biochem. J.* **293**, pp. 781-788).

Said xylanase promoter-operator nucleotide sequences comprise at least one 5 base pairs pattern : 5'-CGAAA-3'.

Preferably, said xylanase promoter-operator nucleotide sequence is the *Streptomyces sp.* strain EC3 xlnC

xylanase promoter-operator nucleotide sequence SEQ ID NO 2 also described by Giannotta F. et al. (*FEMS Microbiol. Letters* **142**, pp. 91-97 (1996)).

According to a preferred embodiment of the present invention, the isolated and purified nucleotide sequence according to the invention is a (DNA) sequence which presents more than 60%, advantageously more than 80%, preferably more than 90%, and more preferably more than 95%, homology (i.e. sequence identity) with the nucleotide sequence SEQ ID NO 1 or its complementary strand described hereafter.

According to another preferred embodiment of the present invention, said isolated and purified nucleotide sequence corresponds to the nucleotide sequence SEQ ID NO 1 or its complementary strand or a portion thereof; preferably a sequence having more than 100 nucleotides and encoding a peptide which still controls positively and/or negatively the expression of a xylanase promoter-operator nucleotide sequence.

Preferably, said sequence portion comprises at least the nucleotides of SEQ ID NO 3 or any nucleotide sequence encoding for its corresponding peptidic sequence.

According to a further preferred embodiment of the present invention, the terms "a portion of the nucleotide sequence SEQ ID NO 1 or its complementary strand" mean any kind of nucleic acid molecule (DNA, RNA, antisense nucleotide sequence, etc.) which is specific of SEQ ID NO 1, comprises more than 15 nucleotides (such as a probe or one or several primers), and which may be used to identify, reconstitute or block the transcription of said specific isolated and purified nucleotide sequence SEQ ID NO 1 or its complementary strand. Said identification, reconstitution or blocking is obtained with known techniques by the person skilled in the art, such as the

use of antisense RNA, specific labelled probe hybridisation or genetic amplification, preferably by PCR (as described in the US patent 4,965,188) or by LCR (as described by Landgren et al. (*Sciences* **241**, pp. 1077-1080 (1988)).

Therefore, the present invention is also related to any nucleotide sequence which presents an homology (i.e. sequence identity) as above-described with SEQ ID NO 1, SEQ ID NO 3 or their complementary strands, or any nucleotide sequence which preferably allows an hybridization ~~hybridisation~~ with SEQ ID NO 1, SEQ ID NO 3 or their complementary strands under standard stringent hybridisation conditions, and which may encode the same or a similar amino-acid sequence due to the redundancy of the genetic code.

Exemplary standard stringent hybridization ~~hybridisation~~ conditions are as follows : hybridization ~~hybridisation~~ at 40 °C in 50% formamide, 5x SSC, 20 mMol sodium phosphate, pH 6.8, washing in 0.2x SSC at 50 °C. Variations in these conditions may occur based on the length and the GC nucleotide content of the sequence to be Hybridized ~~Hybridised~~. Formula standard in the art are approved for determining exact hybridization ~~hybridisation~~ conditions such as the one described by Sambrook et al. (*Molecular Cloning : A Laboratory Manual, Cold Spring Harbor, Laboratory Press, Cold Spring Harbor, New York*, §9.47-9.51 (1989)).

Another aspect of the present invention is related to the amino-acid sequence encoded by said nucleotide sequence, and which present more than 60%, advantageously more than 80%, preferably more than 90%, more preferably more than 95% homology (i.e. sequence identity), with SEQ ID NO 2.

According to another embodiment of the present invention, the amino-acid sequence according to the

invention corresponds to the amino-acid sequence of SEQ ID NO 2 or any portion thereof having preferably more than 50 amino-acids and which is still capable of controlling (positively or negatively) in trans the expression of a xylanase promoter-operator nucleotide sequence.

Preferably, said portion is an amino-acid sequence that comprises at least the amino-acid sequence encoded by the nucleotide sequence SEQ ID NO 3 above-described [SEQ ID NO:4].

It is meant by "controlling (positively and/or negatively) in trans the expression of a xylanase promoter-operator nucleotide sequence", the possibility for any nucleotide sequence 1 or any amino-acid sequence 3 encoded by said nucleotide sequence 1 to induce or reduce (preferably in the presence of the other cofactors 4 such as glucose and/or xylan) the expression of a xylanase promoter-operator nucleotide sequence 2 and obtain thereafter a control upon the cis-activation of a downstream nucleotide sequence 5 (for instance a gene encoding a xylanase enzyme) which is controlled in cis by said xylanase promoter-operator nucleotide sequence. The inducing or reduction of said expression is observed preferably by a positive or a negative modification of said cis-activation (for instance by an increasing or a decreasing of the synthesis of said xylanase enzyme by a cell). Said mechanism is also illustrated in the enclosed Fig. 3.

The present invention is also related to a nucleotide construct 6 comprising the isolated and purified nucleotide sequence 1 according to the invention, linked to a xylanase promoter-operator nucleotide sequence 2 and possibly any homologous or heterologous nucleotide sequence 5 of a gene encoding a xylanase enzyme, which is cis-

activated by said xylanase promoter-operator nucleotide sequence 2.

Another aspect of the present invention is related to the vector 7 comprising said isolated and purified nucleotide sequence 1 or the nucleotide construct 6 according to the invention. Advantageously, said vector 7 is a plasmid comprising the necessary elements (origin of replication ORI) for the transfection of said nucleotide sequence 1 or said nucleotide construct 6 into a cell, preferably into a *Streptomyces sp.* strain.

The vector according to the invention may comprise also other elements, such as a marker (thiostreptone = tsr) for the identification of a possible transformation by the vector according to the invention in said specific cell. The vector according to the invention can be also a shuttle vector comprising the necessary elements for the expression of said shuttle vector in *E. coli* and *Streptomyces sp.*

Another aspect of the present invention is related to the cell such as a gram-positive bacteria, preferably a *Streptomyces* strain, transformed by said vector 7 or by said shuttle vector, which allows the expression of the isolated and purified nucleotide sequence 1 according to the invention controlling the activation of the xylanase promoter-operator nucleotide sequence 2 present in said cell and therefore the transcription of any nucleotide sequence 5 which could be cis-activated by said xylanase promoter-operator nucleotide sequence 2.

The nucleotide construct 6, the vector 7 and/or the cell transformed by said vector as well as specific portions of the isolated and purified nucleotide sequence 1 according to the invention can be advantageously used in several industrial biochemical processes such as production of antibiotics, malting processes of cereals,

preparation of beers, baked or extruded cereals products, for the improving of animal feed stuff and for the improvement of paper and pulp technologies.

The products of the invention, possibly combined with the above-described cofactors, are advantageously present in a bioreactor, and will allow the controlled synthesis of proteins or peptides of interest or possibly avoid or reduce the synthesis of said proteins or peptides by specific cells in the above-identified biochemical industrial processes.

The various aspects of the present invention will be described in details in the enclosed non-limiting examples in reference to the following figures.

**Brief description of the drawings**

Figures 1 to 3 represent the steps for the construction of the vector according to the invention.

**Detailed description of the invention**

The alignment of various nucleotide sequences upstream xylanase gene in the strain *Streptomyces sp.* EC3, shows the presence of three repetitive units of five BP : 5'-CGAAA-3' observed among all xylanase sequences (except in the strain *Actinomadura sp.* which comprises only one repetitive unit).

In the specific strain *Streptomyces sp.* EC3, three boxes in the promoter-operator regions of 390 BP are defined : box 1 (B1) at -200 BP, box 2 (B2) at -210 BP and box 3 (B3) at -350 BP from the ATG codon. The box B3 is extremely conserved between the *Streptomyces* strain. (83% of identity of sequence upon 12 bases).

The identification of the repetitive consensus sequence is presented in the following table 1.

Table 1

| | |
|---|---|
| Cons. B1 | C G A A A C T G T T G A [SEQ ID NO: 7] |
| Cons. B2 | T T T C C G A A A G T T T G C C [SEQ ID NO: 8] |
| Cons. B3 | T C G A A A C T T T C G [SEQ ID NO: 9] |
| Consensus | t **C G A A A** g c c [SEQ ID NO: 10] |

However, it seems that said consensus nucleotide sequence (TXXCGAAAXXGXCXC [SEQ ID NO: 10]) is not present in other known xylanase nucleotide sequence of other bacteria such as *Bacillus* strains.

The Inventors have discovered that the proteinic trans-activation factor according to the invention affects the regulation of said specific portions (B3 > B2 > B1) of the xylanase promoter-operator nucleotide sequence of the *Streptomyces sp.* EC3.

The Inventors have also discovered a modification of the trans factor affinity for the B2 box in repression and induction.

Repression : B3 > B2 > B1

Induction : B3 >> B2 = B1

Additional competitive experiments have identified as a preferred fixation site of the trans-activation factor according to the invention, the above-identified specific regions (boxes 3, 2 and 1).

It should be noted also that the above-described boxes present inverse repeated sequence and a palindrome of 4 BP that seems to be specifically recognised by the proteinic trans-activation factor according to the invention.

Therefore, it seems that the main fixation site of said proteinic trans-activation factor is the box B3, which allows thereafter a fixation upon the box B2 even when a mutation is present in said box B2.

According to said preliminary results, it seems that the control upon the activation of a xylanase gene is based upon operative sites which are specifically recognised by a trans-activation factor which is working as a repressor and which allows the formation of a repressive loop (connection between the B2 and B3 boxes by the trans-activation factor) and avoids the fixation of the RNA-polymerase and thereafter the transcription of a downstream coding nucleotide sequence.

*Genetic identification of the proteinic trans-activation factor and its encoding nucleotide sequence*

The gene coding for xylanase C of *Streptomyces sp.* has been cloned into a multicopy vector which confers positive xylanase phenotype when the host strain is under repression conditions. Repressed clones, which may be a genomic fragment encoding the repressor according to the invention, will be characterised by a wild type phenotype.

Repressors from a genomic bank in the vector pDML614 were isolated.

After plasmid purification, an amplification by PCR allows a raw estimation of the insert size, which is presented in Table 2.

| | | | | |
|---|---|---|---|---|
| PCR conditions : | Step 1 : | | 96 °C | 4 min |
| | Step 2 : | 30 cycles | 94 °C | 30 sec |
| | Step 3 : | | 54 °C | 1 min |
| | Step 4 : | | 72 °C | 3 min 30 sec |
| | Step 5 : | | 72 °C | 10 min |
| | Step 6 : | | 4 °C | |

Table 2 : Size of the insert

| Clone | Size of the PCR product (kb) | Estimated size of the insert (kb) |
|---|---|---|
| S1 | 2,5 | 0,9 |
| S2 | 3,2 | 2,5 |
| S3 | 2,5 | 0,9 |
| S4 | 1,6 | 0,1 |
| S5 | 2,1 | 0,5 |
| S6 | 2,8 | 1,2 |
| pDML614 | 1,6 | 0 |

A sequence of 1022 nucleotides obtained from the clone S6 allows the identification of an open reading frame with several bacterial regulator systems. A first polypeptide of 164 amino acids was identified and the corresponding nucleotide sequence was used as a probe for the isolation of the complete nucleotide sequence SEQ ID NO 1.

The cloning of the carboxy terminal portion was obtained by Southern blotting. 2,5 genomic DNA of *Streptomyces sp.* EC3 are cleaved by several restriction enzymes and have been transferred upon a nylon membrane. A fragment of 720 BP has been amplified and labelled with biotine by PCR, and is used as a probe for the specific ~~hybridisation~~ hybridization of the genomic DNA. A portion of the genomic DNA of *Streptomyces sp.* was cleaved by restriction enzymes and the generated fragments by PCR were introduced in a plasmid pUC for sequencing.

The sequenced nucleotide sequence comprises four open reading frames. The longest open reading frame hereafter called xlnR was implicated in the regulation of the xylanase enzyme, and the corresponding amino-acid sequence was identified by the BLAST software.

The complete isolated and purified nucleotide sequence 1 according to the invention was introduced in a vector 7 having incorporated also a xylanase promoter-operator nucleotide sequence 2 linked to a gene encoding a xylanase enzyme 5. Advantageously, said xylanase promoter-operator nucleotide sequence 2 comprises a poly-linker sequence (nucleotide sequence with several cleaving sites) which improves the insertion of homologous or heterologous sequences. The characteristics of the vector according to the invention were improved by incorporating a specific marker (such as the thiostreptone) which is used for the specific selection of transformed cells.

The vector according to the invention was advantageously a shuttle vector comprising the necessary elements for the transfection of said vector in a *Streptomyces* strain and in *E. coli* (see also U.S. patent 4,992,371 incorporated hereafter by reference).

Preferably, said shuttle vector was prepared according to the method comprising the following steps. The pUC18 polylinker was replaced by a dsDNA fragment containing endonuclease restriction sites and the following dsDNA fragment was entered in a *Hin*dIII-*Eco*RI-digested pUC18 (L08752, Norrander et al., Gene 26, pp. 101-106 (1983)).

dsDNA fragment: SEQ ID NO:6: 5'- AGC TAG GCC TAT CGA TGG CGC GCC AAG CTA GCA ACT TAA GTA GAT CTA ACT AGT CTG CAG CAG AAG CTT AAT ATT TAA TTA AGC GGC CGC AGT ACT CTC GAG CCG CCA TGG GCC CGA TAT CGG TAC CAG GCC T- 3' (or SEQ ID NO:5)
(Endonuclease restriction sites: 5'-*Cla*I-*Asc*I-*Nhe*I-*Afl*II-*Bgl*II-*Spe*I-*Pst*I-*Hin*dIII-*Ssp*I-*Pac*I-*Not*I-*Sca*I-*Xho*I-*Nco*I-*Apa*I-*Eco*RV-*Kpn*I-3').

Thereafter, the streptomycine/spectinomycine resistance gene (Str/Spm) from an omega interposon

(Prentki, P. & Krisch, H.M. Gene 29 pp. 303-313 (1984)) was introduced at the *Hind*III restriction site.

The pUC18 sequence was deleted from the construction and replaced by the *Cla*I-*Kpn*I *Streptomyces* replication origin from the pIJ702 vector (Katz et al., J. Gen. Microbio. 129 pp. 2703-2714).

The construction was achieved to be a shuttle vector: a 1242 bp *Ase*I-*Nde*I DNA fragment, containing the *E. coli* DNA replication origin from the pBR322 vector (J01749, Sutcliffe, J.G., Proc. Natl. Acad. Sci. U.S.A. 75(8), pp. 3737-3741 (1978)) was treated by klenow and introduced in *Eco*RV.

The regulatory sequence *xln*R was introduced in a *Pac*I-*Sca*I-digested vector and the *xln*C structural gene with its promoter in the *Asc*I-*Pst*I restriction sites in order to obtain the shuttle vector "Vpro" according to the invention (see enclosed Fig. 3).

For the analysis of an heterologous expression of foreign~~er~~ genes in *Streptomyces*, the person skilled in the art may refer to the US patent 5,641,663 and the US patent 5,435,730.

Furthermore, the vector according to the invention may also comprise one or more mutations in the xylanase promoter-operator nucleotide sequence 2 in order to improve (increase) a cis-activating by said xylanase promoter-operator nucleotide sequence.

WHAT IN THE CLAIMS

~~CLAIMS~~

Subal > 1. Isolated and purified **bacterial** genetic sequence (1) controlling in trans the expression of a xylanase promoter-operator **bacterial** nucleotide sequence (2).

2. Isolated and purified genetic sequence according to claim 1, being a nucleotide sequence which presents more than 60% homology with the nucleotide sequence SEQ ID NO 1 or its complementary strand.

3. Isolated and purified genetic sequence according to claim 2, which presents more than 80%, preferably more than 90%, more specifically more than 95%, homology with the nucleotide sequence SEQ ID NO 1 or its complementary strand.

4. Isolated and purified genetic sequence according to any one of the preceding claims, being the nucleotide sequence SEQ ID NO 1, its complementary strain or a portion thereof having more than 100 nucleotides and encoding a peptide controlling positively and/or negatively the activation of a xylanase promoter-operator nucleotide sequence.

5. Isolated and purified genetic sequence according to claim 1, being an amino-acid sequence which presents more than 60% homology with SEQ ID NO 2.

6. Isolated and purified genetic sequence according to claim 5, being an amino-acid sequence which presents more than 80%, preferably more than 90%, more specifically more than 95%, homology with SEQ ID NO 2.

7. Isolated and purified genetic sequence according to claim 1, being the amino-acid sequence SEQ ID NO 2 or a portion thereof having more than 50 amino-acids which is capable of controlling positively and/or negatively in trans the expression of a xylanase promoter-operator nucleotide sequence.

8. Nucleotide construct (6) comprising the isolated and purified nucleotide sequence according to any one of the claims 1 to 4, linked to a xylanase promoter-operator nucleotide sequence (2) and possibly a nucleotide sequence (5) which is cis-activated by said xylanase promoter-operator nucleotide sequence (2).

9. Vector (7), preferably a plasmid, comprising the isolated and purified nucleotide sequence (2) according to any one of the claims 1 to 7 or the nucleotide construct (6) according to claim 8.

10. **Bacterial** cell transformed by the vector according to claim 9 and which allows the expression of the isolated and purified genetic sequence according to any one of the claims 1 to 7.

add A2

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 :
C12N 15/76, 9/24, 1/21, C07K 14/36

A1

(11) International Publication Number: WO 00/09717

(43) International Publication Date: 24 February 2000 (24.02.00)

(21) International Application Number: PCT/BE99/00105

(22) International Filing Date: 12 August 1999 (12.08.99)

(30) Priority Data:
60/096,556 14 August 1998 (14.08.98) US

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(72) Inventors; and
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(74) Agents: VAN MALDEREN, Eric et al.; Office Van Malderen, Place Reine Fabiola 6/1, B–1083 Brussels (BE).

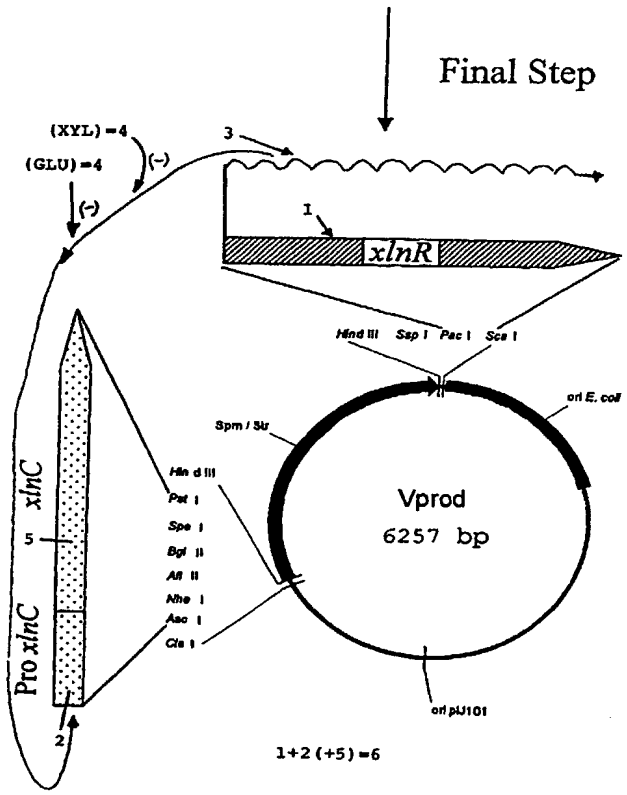
(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

**Published**
*With international search report.*

(54) Title: NUCLEOTIDE AND/OR AMINO–ACID SEQUENCE CONTROLLING THE EXPRESSION OF A XYLANASE PROMOTER–OPERATOR NUCLEOTIDE SEQUENCE

(57) Abstract

The present invention is related to an isolated and purified genetic sequence (1) controlling in trans the expression of a xylanase promoter–operator nucleotide sequence (2).



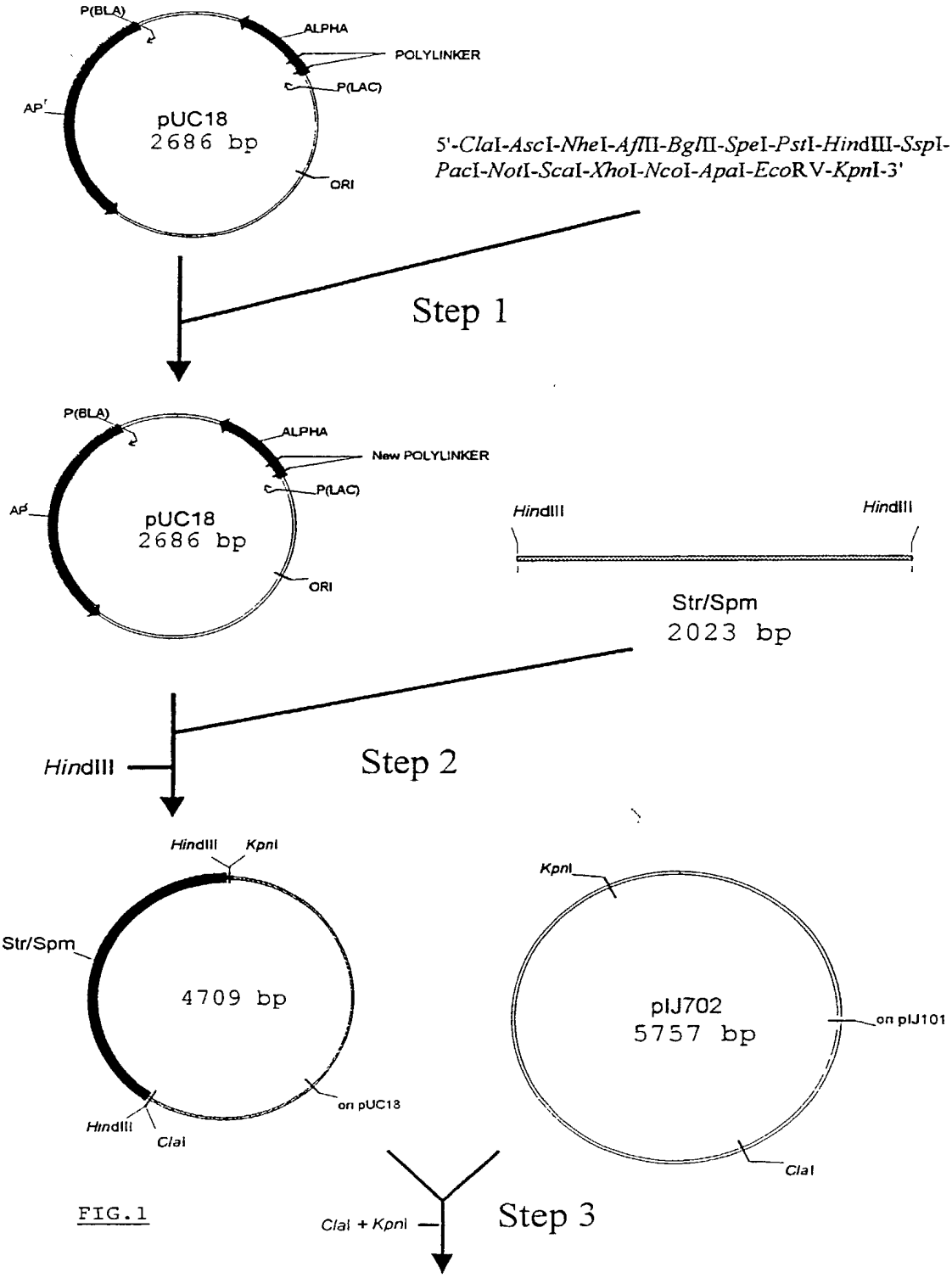

P(BLA)
ALPHA
POLYLINKER
P(LAC)
AP
pUC18
2686 bp
ORI
5'-ClaI-AscI-NheI-AflII-BglII-SpeI-PstI-HindIII-SspI-PacI-NotI-ScaI-XhoI-NcoI-ApaI-EcoRV-KpnI-3'
Step 1
P(BLA)
ALPHA
New POLYLINKER
P(LAC)
AP
pUC18
2686 bp
ORI
HindIII
HindIII
Str/Spm
2023 bp
HindIII
Step 2
HindIII
KpnI
Str/Spm
4709 bp
on pUC18
HindIII
ClaI
KpnI
pIJ702
5757 bp
on pIJ101
ClaI
ClaI + KpnI
Step 3

FIG.1

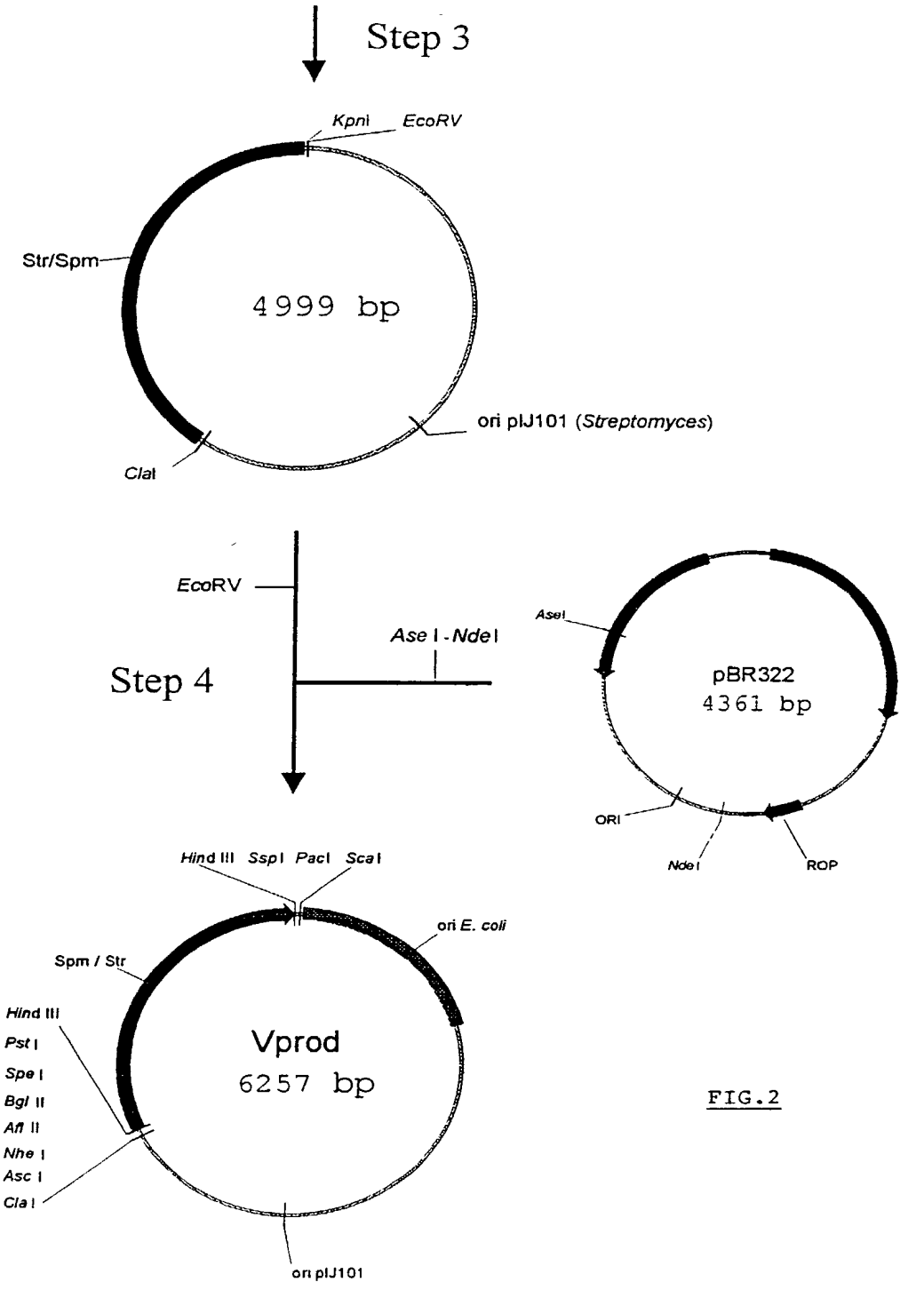

Step 3
KpnI
EcoRV
Str/Spm
4999 bp
ori pIJ101 (Streptomyces)
ClaI
EcoRV
Step 4
Ase I - Nde I
AseI
pBR322
4361 bp
ORI
NdeI
ROP
Hind III SspI PacI ScaI
ori E. coli
Spm / Str
Hind III
Pst I
Spe I
Bgl II
Afl II
Nhe I
Asc I
Cla I
Vprod
6257 bp
ori pIJ101

FIG.2

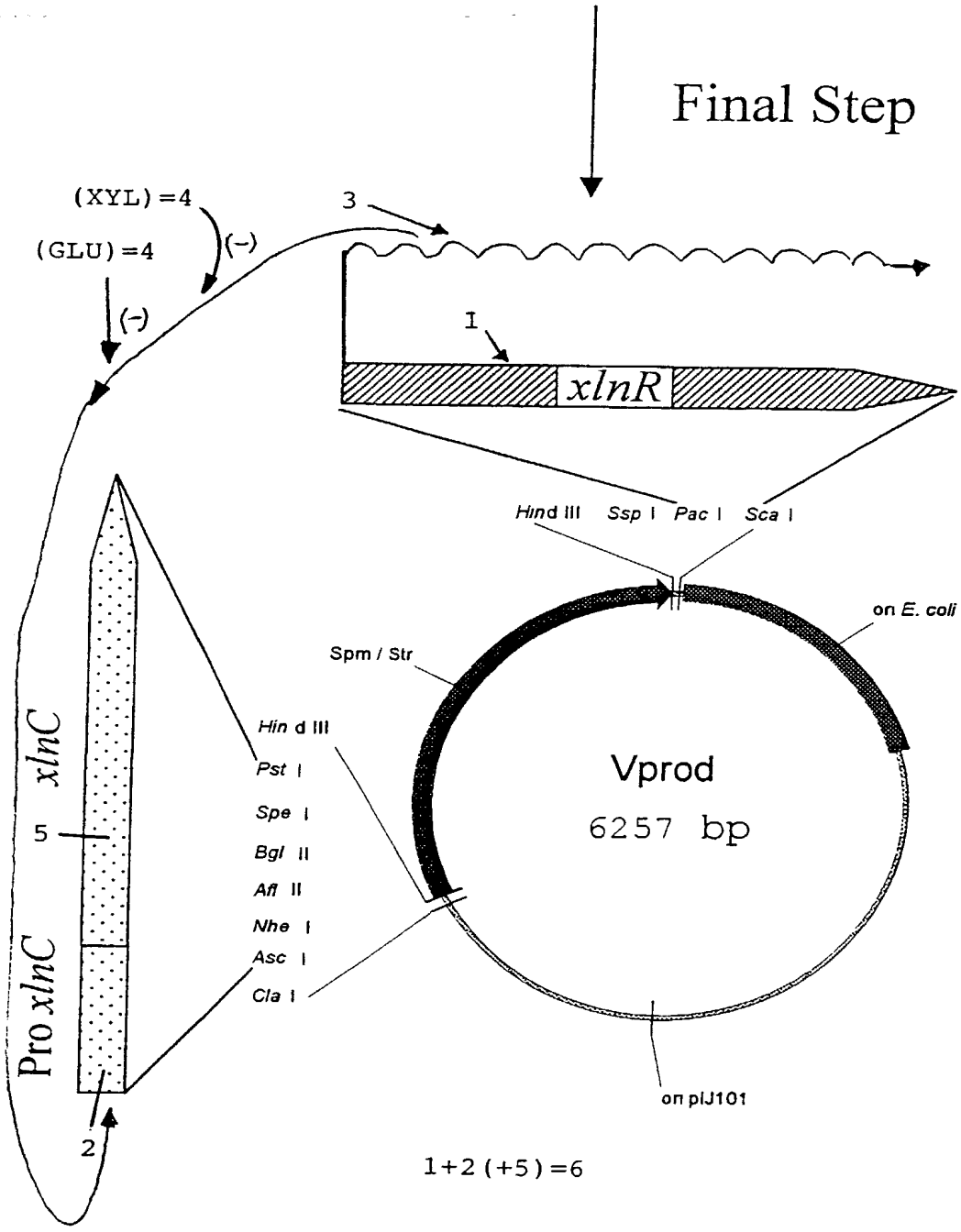

Final Step
(XYL) =4
(GLU) =4
(-)
(-)
3
1
xlnR
Hind III
Ssp I
Pac I
Sca I
on E. coli
Spm / Str
Hin d III
Pst I
Spe I
Bgl II
Afl II
Nhe I
Asc I
Cla I
Vprod
6257 bp
on pIJ101
xlnC
5
Pro xlnC
2
1+2 (+5) =6

FIG.3

P. ULG. 18/US (100)

# *Declaration and Power of Attorney for Patent Application*

# *Déclaration et Pouvoirs pour demandes de brevet*

## French Language Declaration

En tant que l'inventeur nommé ci-après, je déclare par le présent acte que :

As a below named inventor, I hereby declare that :

Mon domicile, mon adresse postale et ma nationalité figurant ci-dessous à côté de mon nom,

My residence, post office address and citizenship are as stated below next to my name,

Je crois être le premier inventeur original et unique (si un seul nom est mentionné ci-dessous), ou l'un des premiers co-inventeurs originaux (si plusieurs noms sont mentionnés ci-dessous) du sujet revendiqué, pour lequel une demande de brevet a été déposée concernant l'invention intitulée :

I believe I am the original, first and sole (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled :

**NUCLEOTIDE AND/OR AMINO-ACID SEQUENCE CONTROLLING THE EXPRESSION OF A XYLANASE PROMOTER-OPERATOR NUCLEOTIDE SEQUENCE.**

et dont les caractéristiques sont fournies ci-joint à moins que la case suivante n'ait été cochée :

the specification of which is attached hereto unless the following box is checked :

○ a été déposé le
sous le numéro de Demande des Etats-Unis ou
sous le numéro de demande internationale
PCT et modifiée le
(le cas échéant).

● was filed on Herewith
as United States Application Number or PCT International Application Number
**PCT/BE99/00105 on 12.08.99 (filed 8/14/99)**
and was amended on (if applicable).

Je déclare par le présent acte avoir passé en revue et pris connaissance du contenu des caractéristiques ci-dessus, revendications comprises, telles que modifiées par tout amendement dont il aura été fait référence ci-dessus.

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

Je reconnais de voir divulguer toute information pertinente à l'examen de cette demande, comme le définit le Titre 37, §1.56 du Code fédéral des réglementations.

I acknowledge the duty to disclose information which is material to patentibility as defined in Title 37, Code of Federal Regulations, § 1.56.

## French Language Declaration

Je revendique par le présent acte avoir la priorité étrangère, en vertu du Titre 35, § 119 du Code des Etats-Unis, sur toute demande étrangère de brevet ou certificat d'inventeur figurant ci-dessous et ai aussi pris connaissance de toute demande étrangère de brevet ou de tout certificat d'inventeur ayant une date de dépôt précédant celle de la demande à propos de laquelle une priorité est revendiquée.

I hereby claim foreign priority under Title 35, United States Code, § 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed.

| Prior foreign applications<br>Demande(s) de brevet antérieure(s) | | Priority claimed<br>Droit de priorité revendiqué | |
|---|---|---|---|
| | | O | O |
| (Number)<br>(Numéro) | (Country)<br>(Pays) | (Day/Month/Year Filed)<br>(Jour/Mois/Année de dépôt) | Yes<br>Oui / No<br>Non |
| **60/096,556** | **US** | **14.08.98** | ● / O |
| (Number)<br>(Numéro) | (Country)<br>(Pays) | (Day/Month/Year Filed)<br>(Jour/Mois/Année de dépôt) | Yes<br>Oui / No<br>Non |
| | | O | O |
| (Number)<br>(Numéro) | (Country)<br>(Pays) | (Day/Month/Year Filed)<br>(Jour/Mois/Année de dépôt) | Yes<br>Oui / No<br>Non |

Je revendique par le présent acte tout bénéfice, en vertu du Titre 35, § 120 du Code des Etats-Unis, de toute demande de brevet effectuée aux Etats-Unis figurant ci-dessous et, dans la mesure où le sujet de chacune des revendications de cette demande de brevet n'est pas divulgué dans la demande américaine préalable, en vertu des dispositions de premier paragraphe du Titre 35, § 112 du Code des Etats-Unis, je reconnais devoir divulguer toute information pertinente à la demande de brevet comme défini dans le Titre 37, § 1.56 du Code fédéral des réglementations, dont j'ai pu disposer entre la date de dépôt de la première demande et la date de dépôt de la demande nationale ou PCT internationale :

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose information which is material to patentibility as defined in Title 37, Code of Federal Regulations, § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application :

| (Application Serial No.)<br>(No. de série de la demande) | (Filing date)<br>(Date de dépôt) | (Statut)<br>(Breveté, en attente, annulé) | (Status)<br>(Patented, pending, abandoned) |
|---|---|---|---|
| (Application Serial No.)<br>(No. de série de la demande) | (Filing date)<br>(Date de dépôt) | (Statut)<br>(Breveté, en attente, annulé) | (Status)<br>(Patented, pending, abandoned) |

Je déclare par le présent acte que toute déclaration ci-incluse est, à ma connaissance, véridique et que toute déclaration formulée à partir de renseignements ou de suppositions est tenue pour véridique; et de plus, que toutes ces déclarations ont été formulées en sachant que toute fausse déclaration volontaire ou son équivalent est passible d'une amende ou d'une incarcération, ou des deux, en vertu de la Section 1001 du Titre 18 du Code des Etats-Unis et que de telles déclarations volontairement fausses risquent de compromettre la validité de la demande de brevet ou du brevet délivré à partir de celle-ci.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful and false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application of any patent issued thereon.

## French Language Declaration

POUVOIRS : En tant que l'inventeur cité, je désigne par la présente l'(les) avocat(s) et/ou agent(s) suivant(s) pour qu'il(s) poursuive(nt) la procédure de cette demande de brevet et traite(nt) toute affaire avec le Bureau des brevets et marques s'y rapportant.
(mentionner le nom et le numéro d'enregistrement)

POWER OF ATTORNEY : As named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and trademark Office connected there with.

(list name and registration number)

Adresser toute correspondance à :

Send Correspondence to :

Adresser tout appel téléphonique à :
*(nom et numéro de téléphone)*

Direct Telephone Calls to :
*(name and telephone number)*

| | |
|---|---|
| Nom complet de l'unique ou premier inventeur | Full name of sole or first inventor<br>**GIANNOTTA Fabrizio** |
| Signature de l'inventeur Date | Inventor's signature X Date<br>8th January 2001 |
| Domicile | Résidence<br>**Rue de la Liberté 38**<br>**B-4020 LIEGE**<br>**BELGIUM** BEL |
| Nationalité | Citizenship<br>**Belgian** |
| Adresse postale | Post Office Address<br>**Rue de la Liberté 38**<br>**B-4020 LIEGE**<br>**BELGIUM** |

(Fournir les mêmes renseignements et la signature de tout co-inventeur supplémentaire)

(Supply similar information and signature for any subsequent joint inventor)

| Nom complet du second co-inventeur, le cas échéant | Full name of second joint inventor, if any<br><br>**~~RIGALI~~ Sébastien** |
|---|---|
| Signature du second inventeur Date | Second inventor's signature X Date<br>8/1/01 |
| Domicile | Residence<br>**Rue Président Kennedy 75**<br>**B-4420 ~~MONTEGNEE~~**<br>**BELGIUM** BEL |
| Nationalité | Citizenship<br>**Belgian** |
| Adresse postale | Post Office Address<br>**Rue Président Kennedy 75**<br>**B-4420 MONTEGNEE**<br>**BELGIUM** |

| Nom complet du troisième co-inventeur, le cas échéant | Full name of third joint inventor, if any<br><br>**~~DUSART~~ Jean** |
|---|---|
| Signature du second inventeur Date | Third inventor's signature X Date<br>8/1/01 |
| Domicile | Residence<br>**Rue d'Esneux 19**<br>**B- 4450 ~~NANDRIN~~**<br>**BELGIUM** BEL |
| Nationalité | Citizenship<br>**Belgian** |
| Adresse postale | Post Office Address<br>**Rue d'Esneux 19**<br>**B- 4450 NANDRIN**<br>**BELGIUM** |

| Nom complet du quatrième co-inventeur, le cas échéant | Full name of fourth joint inventor, if any |
|---|---|
| Signature du second inventeur Date | Third inventor's signature Date |
| Domicile | Residence |
| Nationalité | Citizenship |
| Adresse postale | Post Office Address |

SEQUENCE LISTING

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<120> NUCLEOTIDE AND/OR AMINO-ACID SEQUENCE CONTROLLING THE EXPRESSION OF A XYLANASE PROMOTER-OPERATOR NUCLEOTIDE SEQUENCE

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taggga atg cct gct acc gac gac cgt cgg ccc aag tac cag cgg atc 168
Met Pro Ala Thr Asp Asp Arg Arg Pro Lys Tyr Gln Arg Ile
1 5 10

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Ala Asp Ser Leu Arg Glu Ala Ile Gln Ser Gly Glu Tyr Gly Pro Gly
15 20 25 30

gat cgg ctt ccc ggg gag aac gac ctc atg gcc acg cac ggc gtg gcc 264
Asp Arg Leu Pro Gly Glu Asn Asp Leu Met Ala Thr His Gly Val Ala
35 40 45

cgt atg acg gcc cgg cag gcg ctc ggc gtc ctg cgg gac gag ggc atc 312
Arg Met Thr Ala Arg Gln Ala Leu Gly Val Leu Arg Asp Glu Gly Ile
50 55 60

gcc gaa tcc cgg aag ggc gca ggt gtc ttc gtg cgg gcc ttc cgt ccg 360

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          65                  70                  75

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Leu Arg Arg Arg Gly Ile Gln Arg Leu Ala Arg Asp Gln Trp Gly Asn
      80                  85                  90

gga cgg tcc atc tgg tcg gcg gac atc gag gcc aga gac ctc cgg gtc   456
Gly Arg Ser Ile Trp Ser Ala Asp Ile Glu Ala Arg Asp Leu Arg Val
 95                  100                 105                 110

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Asp Gln Val Ser Val Gly Glu Glu Lys Ala Pro Glu His Ile Gly Ala
                115                 120                 125

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Val Leu Gly Met Ala Ala Glu Glu Val Ala Cys Val Arg Arg Arg Arg
            130                 135                 140

ttc gtc ctg gac ggc aag ccg gtg ctg ctc gcg acg agt tac ctg ccc   600
Phe Val Leu Asp Gly Lys Pro Val Leu Leu Ala Thr Ser Tyr Leu Pro
        145                 150                 155

ctg tcc ctg gtg gcc gga tcc gcc atc agc cga gag gac acc ggg ccg   648
Leu Ser Leu Val Ala Gly Ser Ala Ile Ser Arg Glu Asp Thr Gly Pro
    160                 165                 170

ggc ggt acc tac gcc cgg ctt gcc gaa ctc ggc cac gaa ccg gtg cac   696
Gly Gly Thr Tyr Ala Arg Leu Ala Glu Leu Gly His Glu Pro Val His
175                 180                 185                 190

ttc cgc gag gag atc cgc tca cgc atg ccg tcg ccg gac gag gtg aca   744
Phe Arg Glu Glu Ile Arg Ser Arg Met Pro Ser Pro Asp Glu Val Thr
                195                 200                 205

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Gln Leu Asp Leu Ala Pro Gly Thr Pro Val Ile Leu Ile Cys Arg Thr
            210                 215                 220

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Ala Phe Thr Asp Gln Gly His Pro Val Glu Val Asn Glu Met Thr Leu
        225                 230                 235

gac gcc gct tcc tac gtc ttg gag tac gac ttc gac gcg ggc ccc gag   888
Asp Ala Ala Ser Tyr Val Leu Glu Tyr Asp Phe Asp Ala Gly Pro Glu
    240                 245                 250

ccc gcc tcc ccc ggc gcc gac gcc aca gcg ccc gga gac ccg gcc       933
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  1               5                  10                  15

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            20                  25                  30

Leu Pro Gly Glu Asn Asp Leu Met Ala Thr His Gly Val Ala Arg Met
         35                  40                  45

Thr Ala Arg Gln Ala Leu Gly Val Leu Arg Asp Glu Gly Ile Ala Glu
     50                  55                  60

Ser Arg Lys Gly Ala Gly Val Phe Val Arg Ala Phe Arg Pro Leu Arg
 65                  70                  75                  80

Arg Arg Gly Ile Gln Arg Leu Ala Arg Asp Gln Trp Gly Asn Gly Arg
                 85                  90                  95

Ser Ile Trp Ser Ala Asp Ile Glu Ala Arg Asp Leu Arg Val Asp Gln
            100                 105                 110

Val Ser Val Gly Glu Glu Lys Ala Pro Glu His Ile Gly Ala Val Leu
        115                 120                 125

Gly Met Ala Ala Glu Glu Val Ala Cys Val Arg Arg Arg Arg Phe Val
    130                 135                 140

Leu Asp Gly Lys Pro Val Leu Leu Ala Thr Ser Tyr Leu Pro Leu Ser
145                 150                 155                 160

Leu Val Ala Gly Ser Ala Ile Ser Arg Glu Asp Thr Gly Pro Gly Gly
                165                 170                 175

Thr Tyr Ala Arg Leu Ala Glu Leu Gly His Glu Pro Val His Phe Arg
            180                 185                 190
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Glu Glu Ile Arg Ser Arg Met Pro Ser Pro Asp Glu Val Thr Gln Leu
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Asp Leu Ala Pro Gly Thr Pro Val Ile Leu Ile Cys Arg Thr Ala Phe
210 215 220

Thr Asp Gln Gly His Pro Val Glu Val Asn Glu Met Thr Leu Asp Ala
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Ala Ser Tyr Val Leu Glu Tyr Asp Phe Asp Ala Gly Pro Glu Pro Ala
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Ser Pro Gly Ala Asp Ala Thr Ala Pro Gly Asp Pro Ala
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gcaggtgtct tcgtg 195

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cggtaccagg cctaatt 137

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cggtaccagg cct 133